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(54) Title: STABLE PROTECTION FROM DYSTROPHIC SARCOLEMMAL DEGENERATION AND RESTORATION OF THE SARCOGLYCAN COMPLEX

(57) Abstract

The invention includes compositions, kits, and methods for inhibiting or reversing sarcolemmal damage in a mammal. The methods comprising providing a sarcoglycan protein or a gene vector encoding such a sarcoglycan protein to a muscle cell in the mammal. The sarcoglycan protein is preferably a human delta–sarcoglycan. The compositions, kits, and methods of the invention are useful, for example, for alleviating limb girdle muscular dystrophy in a human patient. The invention also includes a method of delivering a gene vector comprising a nucleic acid which encodes a sarcoglycan protein to a mammal, wherein the method comprises administering one or both of a vascular permeability–enhancing agent such as histamine and a vasodilating agent such as papaverine to the mammal.

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STABLE PROTECTION FROM DYSTROPHIC SARCOLEMMAL DEGENERATION AND RESTORATION OF THE SARCOGLYCAN COMPLEX

FIELD OF THE INVENTION

The field of the invention is gene therapy and muscular dystrophy.

BACKGROUND OF THE INVENTION

Muscular dystrophies are a group of inherited, progressive muscle-weakening disorders. A variety of muscular dystrophies are recognized, including Duchenne muscular dystrophy and limb-girdle muscular dystrophy (LGMD). Variants of the muscular dystrophies have also been recognized, such as LGMD variants 2D, 2E, 2C, and 2F.

Certain of the muscular dystrophies have been associated with abnormalities of one or more proteins of the sarcolemma. For example, Duchenne muscular dystrophy has been associated with abnormalities in a gene encoding a protein designated dystrophin. In humans not afflicted with Duchenne muscular dystrophy, dystrophin is expressed in the sarcolemma in association with numerous other proteins. This multi-protein sarcolemmal assembly is designated the dystrophin-associated protein complex.

Extraction of muscle membrane preparations under non-denaturing conditions has allowed identification of several additional proteins bound to dystrophin. These proteins are collectively known as the dystrophin-associated protein complex. Four of these proteins can be can be dissociated from the others, and have been designated alpha-, beta-, gamma-, and delta-sarcoglycan. These four proteins collectively comprise the sarcoglycan complex. Abnormalities in the genes encoding these four proteins have been implicated in LGMD 2D, 2E, 2C, and 2F, respectively (Bonnemann et al., 1996, Curr. Op. Pediat. 8:569-582). For example, deficiency of the entire sarcoglycan complex has been attributed to a deletion involving the delta-

sarcoglycan (LGMD 2F(1)) promoter in a cardiomyopathic hamster (strain BIO 14.6) model of LGMD (Nigro et al., 1997, Human Mol. Gen. 6:601-607; Sakamoto et al., 1997, Proc. Natl. Acad. Sci. USA 94:13873-13878).

The physiological function of the sarcoglycan complex is unknown. However, epidermal growth factor-like modules in the primary structures of three of its four component proteins suggest a role in ligand-receptor interaction. In transgenic *mdx* mice, the dystrophin isoform designated Dp71 is capable of stabilizing the sarcoglycan complex without preventing progressive muscle degeneration in the diaphragms of these animals (Cox et al., 1994, Nature Genet. 8:333-339; Greenberg et al., 1994, Nature Genet. 8:340-344; Stedman et al., 1991, Nature 352:536-539). These results indicate that presence of the sarcoglycan complex is necessary but not sufficient for full physiological function of the dystrophin-glycoprotein complex.

There is presently no accepted therapy for treatment of muscular dystrophies. Exercise and certain pharmaceutical agents such as prednisone have been reported to extend the duration and quality of motor capacity in patients afflicted with a muscular dystrophy. However, exercise and prednisone merely slow the deterioration of muscular control and strength in muscular dystrophy patients; they do not treat the underlying causes of the disorders. In view of the terrible mortality and morbidity associated with muscular dystrophies, compositions and methods for treating the underlying causes of muscular dystrophies are desperately needed.

The present invention provides such compositions and methods.

SUMMARY OF THE INVENTION

The invention relates to a method of inhibiting sarcolemmal damage in a mammal. The method comprises providing a sarcoglycan protein to a muscle cell in the mammal. Sarcolemmal damage is thereby inhibited. In one embodiment, the sarcoglycan protein is provided to the muscle cell by providing a nucleic acid to the muscle cell. The nucleic acid has a portion encoding the sarcoglycan protein. Upon providing the nucleic acid to the muscle cell, the sarcoglycan protein is expressed from the nucleic acid. The nucleic acid may, for example, be part of a gene vector, such as

one selected from the group consisting of a naked nucleic acid gene vector, a gene vector comprising a nucleic acid and a polycation, and a virus gene vector.

Adeno-associated virus gene vectors are preferred. For example, in one embodiment, the adeno-associated virus gene vector encodes a human sarcoglycan protein selected from the group consisting of alpha-sarcoglycan, beta-sarcoglycan, gamma-sarcoglycan, and delta-sarcoglycan. The portion encoding the sarcoglycan protein may be operably linked with a promoter/regulatory region selected from the group consisting of a human constitutive promoter/regulatory region, a human skeletal-muscle-specific promoter/regulatory region, a human sarcoglycan promoter/regulatory region, and a viral promoter/regulatory region. The human skeletal-muscle-specific promoter/regulatory region may, for example, be a human skeletal muscle creatine phosphokinase promoter/regulatory region, a constitutive promoter/regulatory region of a gene which is ordinarily expressed in a human skeletal muscle cell, or a constitutive promoter/regulatory region of a gene which is ordinarily expressed in a human non-skeletal muscle cell. The viral promoter/regulatory region may, for example, be a cytomegalovirus promoter or a cytomegalovirus immediate early promoter. In a preferred embodiment, the adeno-associated virus gene vector is a rAAVCMVδ-sarc vector, as described herein.

In another embodiment of the method of inhibiting sarcolemmal damage in a mammal, the method further comprises administering to the mammal an agent selected from the group consisting of a vascular permeability-enhancing agent and a vasodilating agent.

The invention also relates to a method of reversing sarcolemmal damage in a mammal. This method comprises providing a sarcoglycan protein to a muscle cell in the mammal. Sarcolemmal damage is thereby reversed.

The invention further relates to a method of alleviating limb-girdle muscular dystrophy in a human patient. This method comprises providing a sarcoglycan protein to a muscle cell in the patient. Limb-girdle muscular dystrophy is thereby alleviated.

The invention still further relates to a pharmaceutical composition for alleviating limb-girdle muscular dystrophy in a human patient. The composition comprises a gene vector comprising a nucleic acid having a portion that encodes a sarcoglycan protein. The sarcoglycan protein may, for example, be a human sarcoglycan protein selected from the group consisting of alpha-sarcoglycan, beta-sarcoglycan, gamma-sarcoglycan, or delta-sarcoglycan. The gene vector may, for example, be an adeno-associated gene vector. The portion that encodes the protein may be operably linked with a promoter/regulatory region, such as one selected from the group consisting of a human constitutive promoter/regulatory region, a human skeletal-muscle-specific promoter/regulatory region, a human sarcoglycan promoter/regulatory region, a cytomegalovirus promoter, a cytomegalovirus immediate early promoter, and a viral promoter/regulatory region.

The invention also relates to use of an adeno-associated gene vector for making a pharmaceutical composition for inhibiting or reversing sarcolemmal damage in a mammal. The adeno-associated gene vector comprises a nucleic acid having a portion encoding a sarcoglycan protein.

The invention further relates to a kit for inhibiting or reversing sarcolemmal damage in a mammal. The kit comprises an adeno-associated gene vector and at least one of a vascular permeabilizing agent and a vasodilating agent. The adeno-associated gene vector comprising a nucleic acid which has a promoter operably linked with a portion that encodes a sarcoglycan protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising Figures 1A, 1B, and 1C, is a trio of images (at 40× magnification) which depict immunofluorescent staining of muscle tissue sections obtained from BIO 14.6 hamsters which were intramuscularly injected with the rAAVCMVδ-sarc gene vector. Immunofluorescent staining using a fluorescently labeled antibody which binds specifically with delta-sarcoglycan protein is depicted in Figure 1A. Immunofluorescent staining using a fluorescently labeled antibody which binds specifically with alpha-sarcoglycan protein is depicted in Figure 1B.

Immunofluorescent staining using a fluorescently labeled antibody which binds specifically with beta-sarcoglycan protein is depicted in Figure 1C.

Figure 2, comprising Figures 2A, 2B, 2C, 2D, and 2E, is a series of images which depict immunofluorescent staining of muscle tissue sections obtained from BIO 14.6 hamsters using a fluorescently labeled antibody which binds specifically with alpha-sarcoglycan protein. Figure 2A is an image (at 40× magnification) of quadriceps muscle obtained from a BIO 14.6 hamster to which rAAVCMVδ-sarc gene vectors were intravenously administered to an isolated hindlimb, as described herein (i.e. in the absence of exogenous histamine or papaverine). Figure 2B is an image (at 200× magnification) of biceps femoris muscle obtained from a BIO 14.6 hamster to which rAAVCMVδ-sarc gene vectors were intravenously administered to an isolated hindlimb, as described herein. Figure 2C is an image (at 100× magnification) of quadriceps muscle obtained from a BIO 14.6 hamster to which rAAVCMVδ-sarc gene vectors were intravenously administered to an isolated hindlimb, as described herein. Figure 2D is an image (at 100× magnification) of semimembranosus muscle obtained from a BIO 14.6 hamster to which rAAVCMVδ-sarc gene vectors were intravenously administered to an isolated hindlimb, as described herein. Figure 2E is an image (at 100× magnification) of gastrocnemius muscle obtained from a BIO 14.6 hamster to which rAAVCMVδ-sarc gene vectors were intravenously administered to an isolated hindlimb, as described herein.

Figure 3 is an illustration of the rodent hindlimb circulatory isolation procedure described herein. Roman numerals indicate the following muscles: I, quadriceps; II, adductors and medial hamstrings; III, gastrocnemius and soleus; and IV, tibialis anterior.

DETAILED DESCRIPTION

Limb-Girdle Muscular Dystrophies (LGMDs) 2C-F are a family of autosomal recessive diseases caused by defects in sarcoglycan genes. The BIO 14.6 hamster is an art-recognized model for LGMD caused by a primary deficiency in delta-

sarcoglycan. Sarcolemmal disruption occurs in this animal model during forceful skeletal and cardiac muscle contraction.

The invention is based on the observation that recombinant adeno-associated virus (rAAV) vectors which comprise a nucleic acid encoding a sarcoglycan can be used to induce efficient and stable expression of delta-sarcoglycan when one of these recombinant vectors is provided to a myocyte of a mammal afflicted with LGMD. The invention is supported by experimental observations of genetic reconstitution of BIO 14.6 hamsters by delivery of delta-sarcoglycan by means of recombinant adeno-associated virus vectors. The vectors may, for example be injected directly into muscle or perfused into muscle by providing the vectors to the bloodstream and by further providing one or both of a vascular permeabilizing agent and a vasodilating agent to the bloodstream.

Thus, the invention includes an effective gene therapy treatment for LGMD in humans and other mammals.

Definitions

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulator sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living human cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living human cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living human cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

By describing two polynucleotides as "operably linked" is meant that a single-stranded or double-stranded nucleic acid moiety comprises the two polynucleotides arranged within the nucleic acid moiety in such a manner that at least one of the two polynucleotides is able to exert a physiological effect by which it is characterized upon the other. By way of example, a promoter operably linked with the coding region of a gene is able to promote transcription of the coding region.

As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property by which it is characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

As used herein, the term "affected cell" refers to a cell of a subject afflicted with a disease or disorder, which affected cell has an altered phenotype relative to the same cell a subject not afflicted with a disease or disorder.

Description

The invention is based on the discovery that sarcolemmal degradation attributable to LGMD may be inhibited or reversed (i.e. "rescued") by providing a recombinant adenoassociated virus (rAAV) vector comprising a nucleic acid encoding a sarcoglycan gene to the affected muscle cells. Various forms of LGMD have been identified, the various forms corresponding to loss or inhibition of normal expression of one or more sarcoglycan genes. The sarcoglycan gene provided to the muscle cells using the rAAV vector is preferably the sarcoglycan gene associated with the form of LGMD with which the subject is afflicted. By way of example, a gene encoding human delta-sarcoglycan may be provided to affected muscle cells of a human afflicted with LGMD type 2F. Alternatively, the sarcoglycan protein may be provided to the sarcoglycan complex of an affected cell by providing the protein itself to the cell.

When the sarcoglycan protein is provided to a cell by providing a gene vector encoding the protein to the cell, the gene vector may be substantially any gene vector which can be used to transfer a gene into a muscle cell. For example, the gene vector may be selected from the group consisting of a naked nucleic acid gene vector, a gene vector comprising a nucleic acid and a polycation, and a virus gene vector. However, as described herein, the identity of the gene vector and the manner in which the vector is delivered can have a tremendous effect on the efficiency with which muscle cells are transduced by the gene vector (i.e. caused to express a protein encoded by a nucleic acid of the gene vector). As noted in the Example herein and in International Patent Application PCT/US98/27072, merely providing an rAAV vector encoding a sarcoglycan to the bloodstream which supplies a muscle tissue is not a highly efficient way to transduce the cells of the muscle tissue. In contrast, pre-treating a muscle tissue (e.g. one isolated from the systemic circulation of the subject) with one or both of a vasodilating agent (e.g. papaverine) and a vascular permeability-enhancing agent (e.g. histamine) concurrently with or prior to providing the vector to the muscle tissue can tremendously increase the efficiency with which the muscle tissue is transduced. Nonetheless, the invention includes all methods of transducing muscle cells with a gene vector, whether the methods are highly or poorly efficient.

In a preferred embodiment, the gene vector used to provide one or more sarcoglycan proteins to the muscle cell(s) is an rAAV gene vector. The sarcoglycan protein may be any vertebrate sarcoglycan protein, but is preferably a mammalian sarcoglycan, and more preferably a human sarcoglycan protein, particularly when the compositions and methods of the invention are intended to be used for treatment of a human afflicted with LGMD. When the human is afflicted with LGMD type 2C, the sarcoglycan is preferably alpha-sarcoglycan. When the human is afflicted with LGMD type 2E, the sarcoglycan is preferably beta-sarcoglycan. When the human is afflicted with LGMD type 2C, the sarcoglycan is preferably gamma-sarcoglycan. When the human is afflicted with LGMD type 2F, the sarcoglycan is preferably delta-sarcoglycan. It is recognized that, particularly in severe instances of LGMD, normal expression of more than one sarcoglycan protein is inhibited or absent, in which instance it is

necessary to provide more than one sarcoglycan protein to the affected muscle cells. More than one sarcoglycan protein may be provided, for example, as a mixture of proteins, by means of a gene vector comprising a nucleic acid encoding more than one sarcoglycan, or by means of a plurality of gene vectors, each comprising a nucleic acid encoding a single sarcoglycan.

When the sarcoglycan protein is provided to a muscle cell in the form of a gene vector comprising a nucleic acid having a portion which encodes a sarcoglycan, the nucleic acid preferably has a promoter/regulatory region operably linked with the portion of the nucleic acid which encodes the sarcoglycan. The promoter/regulatory region may, for example, be a constitutive promoter/regulatory region, an inducible promoter/ regulatory region, a tissue-specific promoter/regulatory region. By way of example, the promoter/regulatory region may be a human constitutive promoter/regulatory region, a human skeletal-muscle-specific promoter/regulatory region, or a viral promoter/regulatory region (e.g. the cytomegalovirus immediate early promoter). An example of an appropriate human skeletal-muscle-specific promoter/regulatory regions is a human skeletal muscle creatine phosphokinase promoter/regulatory region.

In an important embodiment useful for treatment of LGMD type 2F, the gene vector is a rAAVCMVδ-sarc vector, as described herein in the Example.

The invention encompasses the preparation and use of medicaments and pharmaceutical compositions comprising, as an active ingredient, either or both of a sarcoglycan protein and a gene vector comprising a nucleic acid which encodes a sarcoglycan protein. Such a pharmaceutical composition may consist of the active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. Administration of one of these pharmaceutical compositions to a subject is useful for inhibiting or reversing sarcolemmal degradation in muscle cells in the subject, as described elsewhere in the present disclosure. The active ingredient may be present in the pharmaceutical composition in the form of a physiologically

acceptable ester or salt, such as in combination with a physiologically acceptable cation or anion, as is well known in the art.

As used herein, the term "pharmaceutically acceptable carrier" means a chemical composition with which the active ingredient may be combined and which, following the combination, can be used to administer the active ingredient to a subject.

As used herein, the term "physiologically acceptable" ester or salt means an ester or salt form of the active ingredient which is compatible with any other ingredients of the pharmaceutical composition and which is not deleterious to the subject to which the composition is to be administered.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to mammals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various mammals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans, other primates, and mammals including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, and dogs.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for parenteral,

topical, or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. The active agent should be provided to the muscle cell(s) in an amount effective to form stable sarcoglycan complexes with sarcoglycans endogenously expressed by the cell(s). Thus, when the active agent is provided in the form of a sarcoglycan protein, the amount of the protein should be sufficient to make up the differential between the amount of the sarcoglycan produced by normal cells and the amount of the sarcoglycan produced by the dystrophic cells of the subject. When the active agent is provided in the form of a gene vector, the vector should be provided in an amount sufficient to induce expression of the sarcoglycan in the affected cells to at least about the level of expression in normal (i.e. non-dystrophic) muscle cells.

In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents. Particularly contemplated additional agents include, for example, vasodilating agents such as papaverine and vascular permeability-enhancing agents such as histamine.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection and intravenous, intraarterial, or kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules, in multi-dose containers containing a preservative, or in single-use devices for auto-injection or injection by a medical practitioner. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents,

wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Formulations suitable for topical administration include, but are not limited to, liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient, although the concentration of the active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and

described, for example in Genaro, ed., 1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, which is incorporated herein by reference.

It is understood that the ordinarily skilled physician or veterinarian will readily determine and prescribe an effective amount of the compound to inhibit or reverse sarcolemmal degradation in muscle cells in the subject. In so proceeding, the physician or veterinarian may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. It is further understood, however, that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the severity of the sarcolemmal degradation being inhibited or reversed.

Another aspect of the invention relates to a kit comprising a pharmaceutical composition of the invention and an instructional material. As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which is used to communicate the usefulness of the pharmaceutical composition of the invention for inhibiting or reversing sarcolemmal degradation in a subject. The instructional material may also, for example, describe an appropriate dose of the pharmaceutical composition of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains a pharmaceutical composition of the invention or be shipped together with a container which contains the pharmaceutical composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the pharmaceutical composition be used cooperatively by the recipient. The kit may also comprise a vascular permeability-enhancing agent such as histamine, a vasodilating agent such as papaverine, or both.

As described herein, the sarcoglycan protein or gene vector encoding it may be provided to muscle cells of a mammal such as a human using the transvascular delivery method described in International Patent Application PCT/US98/27072. This

method comprises providing a vascular permeability-altering agent to a blood vessel associated with the muscle tissue to increase the permeability of the endothelial layer of the vessel and providing the gene vector to the vessel, whereby the vector is delivered to the muscle tissue through the endothelial layer of the vessel. The muscle tissue may optionally be isolated from systemic circulation. The vascular permeability-altering agent may be provided to the vessel simultaneously with the vector, or may be provided to the vessel before or after the vector is provided. Preferably, the vascular permeability-enhancing agent is histamine or VEGF. The concentration of the vascular permeability-enhancing agent which is used in the method depends on the identity of the agent, but must be sufficient to enhance the permeability of the blood vessel, such that after exposure to the agent, the vessel has a greater permeability than it does before exposure to the agent. Useful concentrations of vascular permeability-enhancing agents are known in the art.

In another embodiment, this method of the invention further comprises providing a vasodilating agent to the vessel, preferably prior to providing the sarcoglycan or gene vector to the vessel, and also preferably prior to providing the vascular permeability-enhancing agent. The vasodilating agent may be provided before, during, or after provision of the composition of the invention. The concentration of the vasodilating agent is not critical, although it must be sufficiently great to induce vasodilation in the vessel. Numerous vasodilating agents are known in the art (e.g. papaverine, nimodipine, hydralazine, nitric oxide, epoprostenol, tolazoline, amrinone, milrinone, nitroglycerine, isosorbide dinitrate, isosorbide mononitrate, and other organic nitrate compounds), as are the concentrations of those agents which are useful for promoting vasodilation. It is contemplated that a higher concentration of the vasodilating agent may be used when the mammal to which the vasodilating agent is administered is subjected to mechanical circulatory support to compensate for the physiological side effects of the vasodilating agent. The use of papaverine in the method of the invention is preferred.

The invention is now described with reference to the following Example. This Example is provided for the purpose of illustration only, and the

invention should in no way be construed as being limited to this Example, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example

BIO 14.6 hamsters are an art-accepted model of LGMD in humans. A deletion involving the delta-sarcoglycan (LGMD 2F(1)) promoter results in secondary deficiency of the entire sarcoglycan complex in these hamsters (Nigro et al., 1997, Human Mol. Gen. 6:601-607; Sakamoto et al., 1997, Proc. Natl. Acad. Sci. USA 94:13873-13878). The experiments presented in this Example indicate that sarcolemmal disruption occurs in this animal model during forceful skeletal and cardiac muscle contraction. These experiments also quantitatively demonstrate that adult BIO 14.6 hamster muscle fibers transformed using an adeno-associated virus vector express recombinant human delta-sarcoglycan and are stably protected from acute sarcolemmal disruption. The delta-sarcoglycan-expressing fibers exhibit dose-dependent rescue of the other components of the sarcoglycan complex without accompanying toxicity or myositis.

The materials and methods used in the experiments presented in this Example are now described.

Muscle preparation and assessment of acute sarcolemmal damage

Adult hamsters were used in these experiments in order to ensure that the hamsters did not exhibit the immunological non-responsiveness to viral antigens which is characteristic of neonatal hamsters (DeMatteo et al., 1997, J. Virol., 71:5330-5335). In human patients afflicted with LGMD, myodegeneration and endomysial fibrosis are likely to have occurred prior to diagnosis. The use of adult hamsters more nearly approximated such patients, because adult hamsters are more likely than neonatal hamsters to exhibit myodegeneration with endomysial fibrosis.

At necropsy, tibialis anterior muscles were atraumatically isolated from BIO 14.6 or F1B Syrian hamsters, and the muscles were placed in individual aliquots of oxygenated Ringer's solution supplemented with 10 millimolar glucose and 25 millimolar HEPES, pH 7.5.

The isolated muscles were mounted between a stationary post and a servo motor lever arm (Cambridge Technology, Watertown, MA) in a custom flow-through chamber device. The device is illustrated in Figure 4. The chamber was a rectangular reservoir having dimensions of approximately 2 × 3 × 6 centimeters, and had inflow and outflow ports for recirculation of oxygenated Ringers solution. At one end of the chamber is a cylinder approximately 4 millimeters in diameter and 1 centimeter in length used to mount one end of the muscle. At the other end of the chamber is a mount for a servo motor (Data Translation, Marlboro, MA) having a servo motor arm. After a muscle mounted between the cylinder and servo motor arm, platinum electrodes were inserted along opposite sides of the muscle to allow field stimulation. The superfusate was isotonic saline buffered with 25 millimolar HEPES (pH 7.5) and bubbled with oxygen in a Radnoti (Radnoti Glass Technology, Inc. Monrovia, CA) oxygenator at 23°C. Platinum electrodes on each side of the muscle enabled field stimulation from a Grass stimulator (Grass Instruments, Inc., West Warwick, RI, Model S48). The motor and stimulator were under computer control using custom software. The software was analogous to that described by Lankford et al. (1995, J. Clin. Invest. 95:1409-1414). Each muscle was stimulated while developed force was monitored and the muscle lengthened to find peak developed force (designated L_0).

The experimental contractile protocol consisted of inducing 5 twitches followed by a tetanus, which was induced by stimulation of each muscle at 60 Hz, for 0.9 second. It was observed that contractile force plateaued by 0.3 second, and that a ramp stretch began after 0.5 second of tetanus. The ramp stretch was 30% of initial muscle length and occurred at a velocity of 0.75 muscle lengths per second for a ramp duration period of 0.4 second. After the tetanus ended, original muscle length was resumed over a period of about 20 seconds. This protocol was repeated every 80 seconds, a total of 6 times.

After the experimental contractile protocol was completed, muscles were dismounted and maintained in pH 7.5 Ringer's solution supplemented with 25

millimolar HEPES buffer and 1% (w/v) procion orange. This solution was oxygenated for 3 hours. Following this treatment, the muscles were blotted dry and frozen in isopentane/liquid nitrogen.

Construction of the recombinant AAV vector

A full length cDNA encoding human delta-sarcoglycan was obtained by PCR amplification of a human fetal muscle cDNA library, as described (Stedman et al., 1988, Genomics 2:1-7). Amplification primers having the sequences

5'-CGGTTTGTGA AACGGACAAG AG-3' (SEQ ID NO: 1) and

5'-GCCAACAATG TCCACTATG-3' (SEQ ID NO: 2)

were used, based on sequence data obtained from Genebank accession no. X95191 (Nigro et al., 1996, Human Mol Genet. 5:1179-1186). Amplification was performed using *Pwo* polymerase (obtained from Boehringer Mannheim, Indianapolis, IN) for 40 cycles. Each cycle comprised maintaining the reaction mixture at 95°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 60 seconds.

Amplification products were gel purified and blunt-end ligated into plasmid pBC (Bluescript™, Stratagene, La Jolla, CA) which had been linearized using restriction endonuclease *Eco*RV. The cDNA was sequenced to confirm the integrity of the open reading frame. Plasmid pSub201(+) (Samulski et al., 1987, J. Virol. 61:3096-3101) was digested using restriction endonuclease *Xba*I in order to allow replacement of the *rep* and *cap* sequences with a "stuffer" sequence derived from the 594 base pair *Xba*I fragment beginning at nucleotide 5475 of cDNA Genebank accession no. X13988 (Eller et al., 1989, Nucl. Acids Res. 17:3591-3592). Sufficient "stuffer" DNA was included in the construct to fill the AAV vector capsid. The "stuffer" DNA that was used included part of the 3'-most 500-1000 base pairs of the human embryonic myosin heavy chain cDNA, which is transcriptionally inactive and does not have known transcription factor binding properties in mammalian cells. The delta-sarcoglycan cDNA was cloned into a CMV transcriptional cassette which was subsequently cloned as a *Sal*I fragment into an *Xho*I site situated within the stuffer sequence. The resulting plasmid was used to produce a purified preparation of recombinant AAV (herein

designated "rAAVCMVδ-sarc") as described (Fisher et al., 1996, J. Virol. 70:520-5326) having a titer of 1×10^{12} genome equivalents per milliliter.

Intramuscular injection of rAAVCMVδ-sarc

Cardiomyopathic (BIO 14.6) Syrian hamsters (obtained from Biobreeders, Cambridge, MA) were anesthetized using ketamine and xylazine. Five millimeter skin incisions were made over the fibular head in order to expose the proximal tibialis anterior. Intramuscular injection 25-100 microliters of the purified rAAVCMVδ-sarc preparation (i.e. approximately 1×10^{12} genome equivalents per milliliter suspended in 20 mM HEPES buffered saline at pH 7.5) was performed along the length of the muscle using either a Hamilton™ (Reno, NV) syringe (50 microliter capacity; model number 80530) fitted with a 33 gauge needle or a glass micropipette (model number 89502).

Intravascular injection of a recombinant adenovirus or rAAVCMVδ-sarc

The femoral artery and vein in a leg of individual adult Fisher 344 rats and BIO 14.6 hamsters were surgically isolated, and two overlapping 2-0 prolene tourniquets were transmuscularly placed at the level of the proximal thigh, as illustrated in Figure 3. Each rat or hamster was intravenously systemically heparinized at a dose of 100 units per kilogram body weight, and the superficial inferior epigastric vessels were retrogradely cannulated using heat-tapered polyethylene tubes (PE 10, Becton Dickinson, Sparks MD) mounted at the ends to 30 gauge needles.

After tightening the tourniquets, microvascular clamps were placed to occlude the femoral vessels. The hindlimb circulation was then primed by infusing arterially either phosphate buffered saline (PBS, pH 7.4) or 150 micrograms of papaverine in 500 microliters of a solution of 10 millimolar histamine in PBS. Five minutes later, a suspension of either 6×10^{10} particles of a recombinant adenovirus vector (AdCMV1acZ; Kozarsky et al., 1993, Som. Cell Mol. Genet. 5:449-458) or 7×10^{11} particles of rAAVCMVδ-sarc in 500 microliters was infused arterially. The virus vectors were suspended in either PBS or PBS comprising 10 millimolar histamine; in either situation, the infusate was chased by arterially infusing PBS in an amount of 1

milliliter per 100 grams of body weight. This infusion was performed by applying high pressure (20 to 80 psig) to the cannula inlet. However, because the cannula tips have an inner diameter smaller than 100 micrometers, the resistance to flow through the inlet was very high, and hence the pressure drop across the inlet was significant. Intravascular pressure measurement is impractical, owing to the small diameter of the vessels. Nonetheless, infusion ceased before the system reached equilibrium, after a selected volume of infusate i.e. (1 to 2 % of body mass) was infused. Following this perfusion/infusion, the limb circulation was flushed with 3 ml of PBS, and residual histamine and papaverine were thereby flushed into the venous catheter.

The femoral vessels were electrocoagulated distally as the cannulae were withdrawn, the clamps on the femoral vessels and tourniquets were removed, and the incision was closed using a resorbable suture. Tissue specimens obtained from rats were whole mount stained for lacZ activity following perfusion and fixation with 0.2% glutaraldehyde and 2% paraformaldehyde in PBS at necropsy, as described (Sanes et al., 1986, EMBO J. 5:3133-3142). Muscle tissue from BIO 14.6 hamsters was snap-frozen in isopentane and cryosectioned for further analysis.

Histology

Hematoxylin, eosin, and Alizanine Red S staining of BIO 14.6 muscle tissue was performed as described in Brumback et al. (1984, Color Atlas of Muscle Histochemistry, PSG Publ. Co., Littleton, MA).

Dye uptake by muscle fibers of hamsters which had been injected three days earlier with Evans Blue Dye was performed as described (Holt et al., 1998, Mol. Cell) 1:841-848).

Immunohistochemistry

Immunohistochemistry was performed on serial 5 gram cryosections of frozen muscle tissue obtained from BIO 14.6 hamsters. Mouse monoclonal antibodies which bind specifically with alpha- sarcoglycan, beta- sarcoglycan, delta- sarcoglycan, and dystrophin (MAbs NCL-50DAG, NCL-Bsarc, NCL-Dsarc, and NCL-DYS2, respectively) were obtained from Novocastra Laboratories (Newcastle upon Tyne, UK).

Cryosections were stained with 1:100 to 1:200 dilutions of primary antibodies. Donkey anti-mouse FITC (fluorescein isothiocyanate; obtained from Novocastra Laboratories) secondary antibodies were used for immunofluorescence staining. Procion orange (PO) was simultaneously visualized in muscle tissue samples using standard U.V. illumination and an FITC filter.

Total numbers of alpha-sarcoglycan positive (α+) and PO positive (PO+) fibers were assessed by planimetry using high power composite micrographs, and these data were used to determine values for the numbers as a percentage of the total number of fibers per section. In cases of PO staining, deficits were noted centrally in the muscle. These staining deficits appeared to be attributable to inhibited diffusion of PO to the central portions of muscle tissue samples. Cross-sectional area of PO deficient tissue was estimated and the number of muscle fibers in each non-PO-stained section were excluded from the calculations of staining on a percentage-of-fibers basis. Criteria for PO+ and α+ fibers were as previously described for the *mdx* mouse (Petrof et al., 1993, Proc. Natl. Acad. Sci. 90:3710-3714; Deconinck et al., 1996, Proc. Natl. Acad. Sci. USA 93:3570-3574).

The results presented in this Example are now described.

In order to evaluate uptake of an rAAV vector by muscle fibers in adult hamsters, the pattern of muscle injury in hamsters was observed three days after injecting them with Evans Blue dye. Recently injured muscle fibers appeared red when viewed by fluorescence microscopy using a rhodamine filter. Immunostaining of these muscle samples revealed that injured fibers did not express dystrophin, beta-dystroglycan, or spectrin. The proteolysis evident in these fibers indicated that this assay method could not be reliably used to evaluate muscle fiber transformation, because transduced fibers that admitted Evans Blue dye would be scored as sarcoglycan negative.

In BIO 14.6 hamsters which had been injected with Evans Blue dye and permitted overnight (ca. 8 hours) access to a running wheel, sporadic clusters of weakly dye-positive muscle fibers were observed upon necropsy. These fibers also exhibited a

hazy rim of residual dystrophin upon immunostaining. More strongly dye-positive fibers did not exhibit dystrophin immunostaining.

In order to better define the time course of sarcolemmal disintegration, forceful contraction of skeletal muscle obtained from control (i.e. F1B Syrian) or cardiomyopathic (i.e. BIO 14.6) hamsters was induced *ex vivo*. Immediately after inducing contraction, sarcolemmal integrity was assessed using the vital dye designated procion orange, as described (Petrof et al., 1993, Proc. Natl. Acad. Sci. USA 90:3710-3714). Immunological dystrophin staining persisted in essentially all fibers which stained positive with procion orange. For this reason, the procion orange staining assay was used as an indicator of delta-sarcoglycan transduction in subsequent experiments.

Acute sarcolemmal damage was modeled *in vivo* by obtaining muscle tissue samples from BIO 14.6 hamsters after approximately one hour of voluntary wheel running. Evans Blue dye uptake was barely detectable in most dystrophin-positive cells, and sheets of Alizarin Red S-positive fibers were also dystrophin-positive. These results suggest that recent injury is associated with massive calcium influx into muscle fibers. Because voluntary running is sporadic in BIO 14.6 hamsters, the more reproducible *ex vivo* assay described above was used in subsequent experiments to assess the efficacy of gene transfer in muscles that could be atraumatically excised with their origins and insertions intact.

The percentage of muscle fibers taking up procion orange dye was quantitated in control and cardiomyopathic hamster muscles following eccentric contracture at a range of lengthening rates. As with muscle tissue obtained from *mdx* mice (Petrof et al., 1993, Proc. Natl. Acad. Sci. USA 90:3710-3714), the greatest difference between control and dystrophic muscle occurred during tetanic stimulation at lengthening rates between 0.5 and 1.0 muscle lengths per second. At 0.75 muscle lengths per second, the percentage of procion orange-positive fibers was 2.79% in BIO 14.6 hamsters and 0.33% in control hamsters. These data may be expressed as a ratio of percentages equal to (2.79/0.33=) 8.5, and represent the level of protection conferred to the sarcolemma by the native sarcoglycan complex. This value may be used as a

benchmark for the functional assessment of expression of recombinant delta-sarcoglycan in transduced muscle fibers.

A recombinant AAV vector (rAAVCMVδ-sarc) was constructed, and comprised a full-length cDNA encoding human delta-sarcoglycan under the transcriptional control of a cytomegalovirus early-immediate gene transcriptional cassette. This vector was used to transduce muscle fibers in BIO 14.6 hamsters of various ages in order to assess stability of delta-sarcoglycan expression, the volume of tissue in which the construct was expressed, histological evidence of toxicity, and the efficacy of delta-sarcoglycan expression for preventing sarcolemmal degeneration and for restoring the sarcoglycan complex.

Muscle tissue samples obtained from hamsters which were intramuscularly injected with rAAVCMVδ-sarc at 4 weeks of age were examined using the indirect immunofluorescence methods described herein after sacrificing the animals at 10 weeks of age. In these tissue samples, widespread expression of recombinant delta-sarcoglycan was observed with characteristic gradients of sarcolemmal staining intensity centered around injection sites. At those injection sites, sarcoplasmic immunostaining was detected, as depicted in Figure 1A. Serial cryosections which were immunostained for alpha-sarcoglycan and beta-sarcoglycan expression demonstrated a dose-dependent restoration of the sarcoglycan complex in tissue areas remote from the injection site, as depicted in Figures 1B and 1C, respectively. However, saturating levels of exclusively sarcolemmal alpha- and beta-sarcoglycan were observed in fibers which expressed sarcoplasmic delta-sarcoglycan.

Hematoxylin and eosin (H&E) stained sections revealed no evidence of inflammatory cell infiltration around transduced muscle fibers. A gradient of from 100% peripheral myonuclei near the injection site to 100% central myonuclei remote from the injection site was observed. Centrally nucleated muscle fibers are the histological hallmark of recent myodegeneration and regeneration. For myonuclei to assume a peripheral location within a muscle fiber, the fiber must be protected from degeneration. Thus the H&E analysis of this tissue indicates evidence that the recombinant protein protects the muscle fibers.

The rAAVCMVδ-sarc vector was injected into tibialis anterior muscle tissue of a second cohorts of hamsters at 9 weeks of age. Muscle tissue samples obtained from these hamsters 13 weeks after injection (i.e. at 22 weeks of age) were subjected to lengthening, as described herein, at 0.75 muscle lengths per second during tetanic contracture. A two-channel stain for the sarcoglycan complex and uptake of procion orange was performed in order to allow simultaneous scoring of individual fibers for sarcoglycan expression and for protection against sarcolemmal damage.

Muscle tissue samples obtained from each hamster of the second cohort exhibited sustained sarcoglycan expression in the absence of apparent inflammatory cell infiltration. Procion orange uptake was observed in clustered fibers at apparently random sites throughout the muscle cross section. Expression of alpha-sarcoglycan and uptake of procion orange by muscle fibers was quantitatively assessed by counting positively-stained fibers on composite micrographs of muscle tissue samples. These data are listed in Table 1.

Table 1

Strain	n	Age at Inj.(wk)	rAAV-δsarc genome eq.	Age (wks) at necropsy	% α+ fibers[A]	% PO+ fibers[B]	%α+ fibers also PO+[C]	%α- fibers also PO+[D]
BIO 14.6	1	3	2.5×10^9	6	14.1 (355/2502)	ND	ND	ND
BIO 14.6	5	9	2×10^{10}	22	4.4 (766/17427)	2.26	0.52	2.36
BIO 14.6	2	non-injected	-	22	0	2.79	-	2.79
F1B (control)	2	Not Injected	-	22	100	0.33	0.33	-

Notes: [A] Percentage of muscle fibers which stained positive for alpha-sarcoglycan expression.

[B] Percentage of muscle fibers which stained positive for procion uptake.

[C] Percentage of muscle fibers stained positive for both alpha-sarcoglycan expression and procion uptake.

[D] Percentage of muscle fibers which stained negative for alpha-sarcoglycan expression but positive for procion up

[E] Not determined

The data listed in Table 1 indicate that muscle fibers which expressed detectable alpha-sarcoglycan (i.e. fibers in which endogenously-expressed alpha-sarcoglycan was retained in functional sarcoglycan complexes upon expression of delta-sarcoglycan following transduction) were more than four times (i.e. 2.36/0.52) less likely to exhibit sarcolemmal degeneration than muscle fibers in the same muscle tissue sample which did not express detectable alpha-sarcoglycan. These data confirm that transduction of muscle cells with a recombinant AAV vector encoding a sarcoglycan such as human delta-sarcoglycan inhibits sarcolemmal degeneration.

However, transduction effected by injection of a gene vector into skeletal muscle can, as described above, be limited to focal areas surrounding the injection site. Much more efficient transduction of muscle cells may be achieved by using a transvascular method of delivering a gene vector to an extravascular tissue, such as that described in International Patent Application PCT/US98/27072. This method involves providing the gene vector and a vascular permeability-altering agent such as histamine to a blood vessel associated with the extravascular tissue. A vasodilating agent such as papaverine may also be provided to the blood vessel, and these vectors and agents may be provided to the blood vessel under superatmospheric pressure in order to further enhance delivery of the gene vector to the tissue.

Recombinant AAV vectors do not penetrate intact skeletal muscle endothelium, as demonstrated in the following experiments.

The rAAVCMVδ-sarc vector described herein was administered to 8-week-old BIO 14.6 hamsters by high pressure femoral injection into an isolated hindlimb. Neither histamine nor papaverine was co-administered. Quadriceps muscle tissue samples were harvested at necropsy at 25-30 weeks of age. The harvested muscle tissue was immunologically stained, as described herein, to detect expression of delta-sarcoglycan. As indicated in Figure 2A, expression of delta-sarcoglycan from the rAAV vector could not be detected, even when the titer of vector administered to the hamster was as high as 10^{11} particles per gram of tissue.

The transvascular delivery method described in International Patent Application PCT/US98/27072 was used to deliver the rAAVCMVδ-sarc vector

described herein to hindlimb muscle of BIO 14.6 hamsters, as described herein. As described above, the hindlimb circulation was first treated with papaverine and histamine, and the vector was administered following this treatment. As indicated in Figures 2B, 2C, 2D, and 2E, this treatment resulted in efficient transfer of the vector to the biceps femoris (Figure 2B), quadriceps (Figure 2C), semimembranosus (Figure 2D), and gastrocnemius (Figure 2E) muscles of the hamster hindlimb. The homogeneity of the staining in Figures 2B-2E indicates that transvascular delivery of the gene vector resulted in more widespread transduction of muscle fibers than did intramuscular injection, as indicated by the highly localized staining in Figures 1A-1C. Furthermore, as indicated by the mosaiform staining pattern indicated in Figures 2B-2E, demonstrates that transvascular delivery of the gene vector resulted in a sufficiently high multiplicity of infection in the best perfused muscle fibers that supranormal levels of delta-sarcoglycan gene expression were induced. H&E staining of serial cryosections of muscle tissue indicated no evidence of inflammatory cell infiltration into the muscle.

The demonstration herein of dose-dependent rescue of the sarcoglycan complex by recombinant delta-sarcoglycan indicates that a similar phenomenon will occur when any one or more of the other sarcoglycans is expressed in LGMD 2C, 2D, and 2E muscle.

The results presented in this Example demonstrate that a recombinant adenoassociated virus vector comprising a nucleic acid encoding a human sarcoglycan protein may be used to inhibit sarcolemmal degradation and to rescue the sarcoglycan complex in dystrophic muscle cells. Thus, providing such a vector to a muscle cell in a mammal afflicted with limb girdle muscular dystrophy is an effective method for inhibiting dystrophic muscle degradation and for reversing the musculodegenerative effects of limb girdle muscular dystrophy or any other dystrophic disease associated with decreased or no expression of a functional sarcoglycan.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A method of inhibiting sarcolemmal damage in a mammal, said method comprising providing a sarcoglycan protein to a muscle cell in the mammal, whereby sarcolemmal damage is inhibited.

2. The method of claim 1, wherein said sarcoglycan protein is provided to the muscle cell by providing a nucleic acid to the muscle cell, wherein said nucleic acid has a portion encoding said sarcoglycan protein, whereby upon providing said nucleic acid to the muscle cell, said sarcoglycan protein is expressed from said nucleic acid.

3. The method of claim 2, wherein said nucleic acid is part of a gene vector.

4. The method of claim 3, wherein said gene vector is selected from the group consisting of a naked nucleic acid gene vector, a gene vector comprising a nucleic acid and a polycation, and a virus gene vector.

5. The method of claim 4, wherein said virus gene vector is an adeno-associated virus gene vector.

6. The method of claim 5, wherein said adeno-associated virus gene vector encodes a human sarcoglycan protein selected from the group consisting of alpha-sarcoglycan, beta-sarcoglycan, gamma-sarcoglycan, and delta-sarcoglycan.

7. The method of claim 6, wherein said human sarcoglycan protein is a human delta-sarcoglycan protein.

8. The method of claim 6, wherein said portion is operably linked with a promoter/regulatory region selected from the group consisting of a human constitutive promoter/regulatory region, a human skeletal-muscle-specific promoter/regulatory region, a human sarcoglycan promoter/regulatory region, and a viral promoter/regulatory region.

9. The method of claim 8, wherein said human skeletal-muscle-specific promoter/regulatory region is selected from the group consisting of a human skeletal muscle creatine phosphokinase promoter/regulatory region, a constitutive promoter/regulatory region of a gene which is ordinarily expressed in a human skeletal

muscle cell, and a constitutive promoter/regulatory region of a gene which is ordinarily expressed in a human non-skeletal muscle cell.

10. The method of claim 8, wherein said viral promoter/regulatory region selected from the group consisting of a cytomegalovirus promoter and a cytomegalovirus immediate early promoter.

11. The method of claim 5, wherein said adeno-associated virus gene vector is a rAAVCMVδ-sarc vector.

12. The method of claim 1, further comprising administering to the mammal an agent selected from the group consisting of a vascular permeability-enhancing agent and a vasodilating agent.

13. A method of reversing sarcolemmal damage in a mammal, said method comprising providing a sarcoglycan protein to a muscle cell in the mammal, whereby sarcolemmal damage is reversed.

14. A method of alleviating limb-girdle muscular dystrophy in a human patient, said method comprising providing a sarcoglycan protein to a muscle cell in the patient, whereby limb-girdle muscular dystrophy is alleviated.

15. A pharmaceutical composition for alleviating limb-girdle muscular dystrophy in a human patient, said composition comprising a gene vector which comprises a nucleic acid having a portion that encodes a sarcoglycan protein.

16. The pharmaceutical composition of claim 15, wherein said sarcoglycan protein is a human sarcoglycan protein selected from the group consisting of alpha-sarcoglycan, beta-sarcoglycan, gamma-sarcoglycan, and delta-sarcoglycan.

17. The pharmaceutical composition of claim 15, wherein said gene vector is an adeno-associated gene vector.

18. The pharmaceutical composition of claim 14, wherein said portion is operably linked with a promoter/regulatory region selected from the group consisting of a human constitutive promoter/regulatory region, a human skeletal-muscle-specific promoter/regulatory region, a human sarcoglycan promoter/regulatory region, a cytomegalovirus promoter, a cytomegalovirus immediate early promoter, and a viral promoter/regulatory region.

19. Use of an adeno-associated gene vector for making a pharmaceutical composition for inhibiting or reversing sarcolemmal damage in a mammal, wherein said adeno-associated gene vector comprises a nucleic acid having a portion encoding a sarcoglycan protein.

20. A kit for inhibiting or reversing sarcolemmal damage in a mammal, said kit comprising

an adeno-associated gene vector comprising a nucleic acid which has a promoter operably linked with a portion that encodes a sarcoglycan protein; and

at least one of a vascular permeabilizing agent and a vasodilating agent.

Fig. 1A

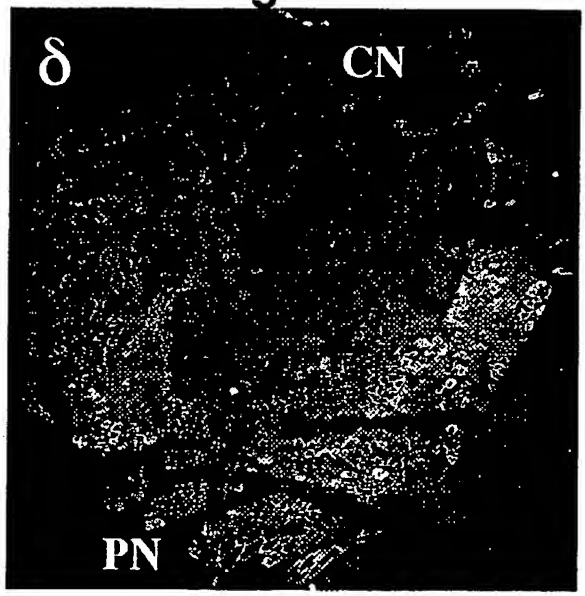


Fig. 1C

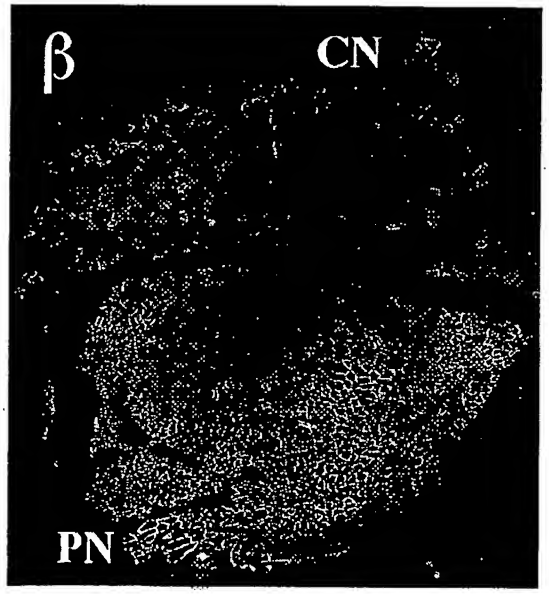
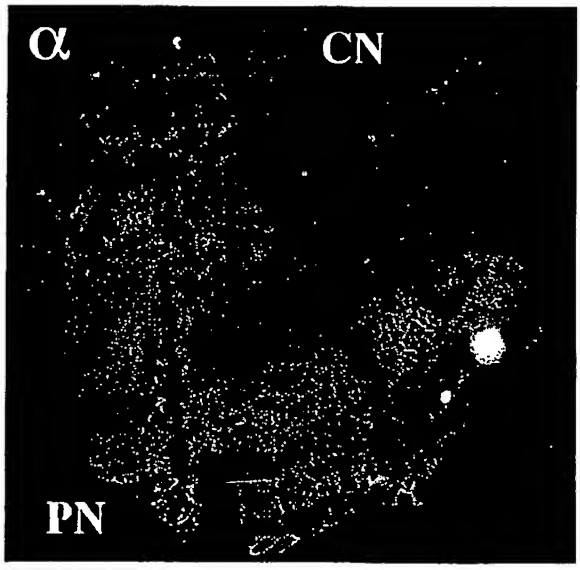


Fig. 1B



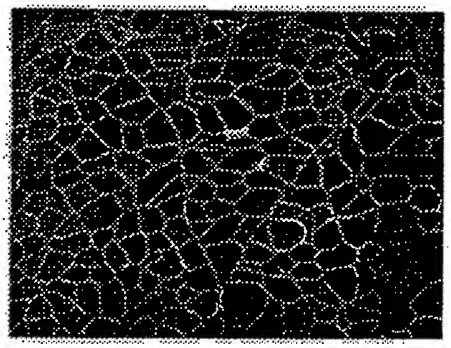


Fig. 2E

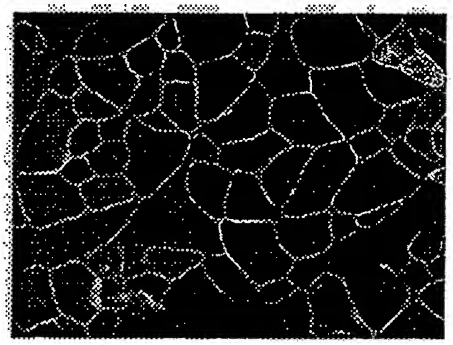


Fig. 2D

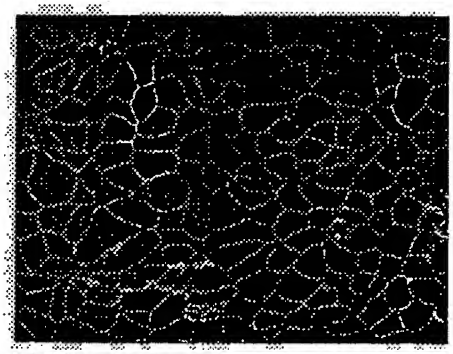


Fig. 2C

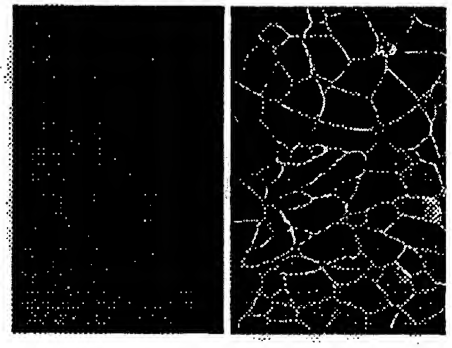
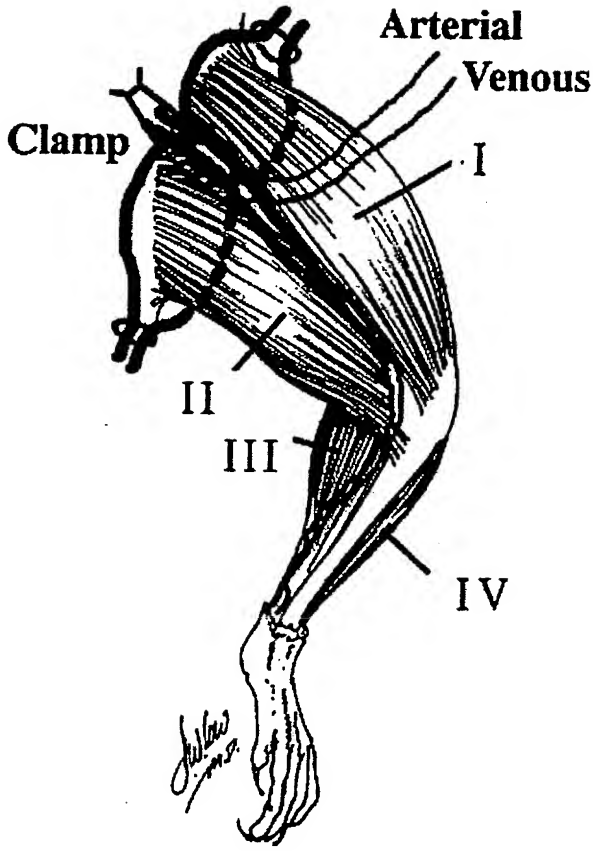


Fig. 2B

Fig. 2A

Fig. 3



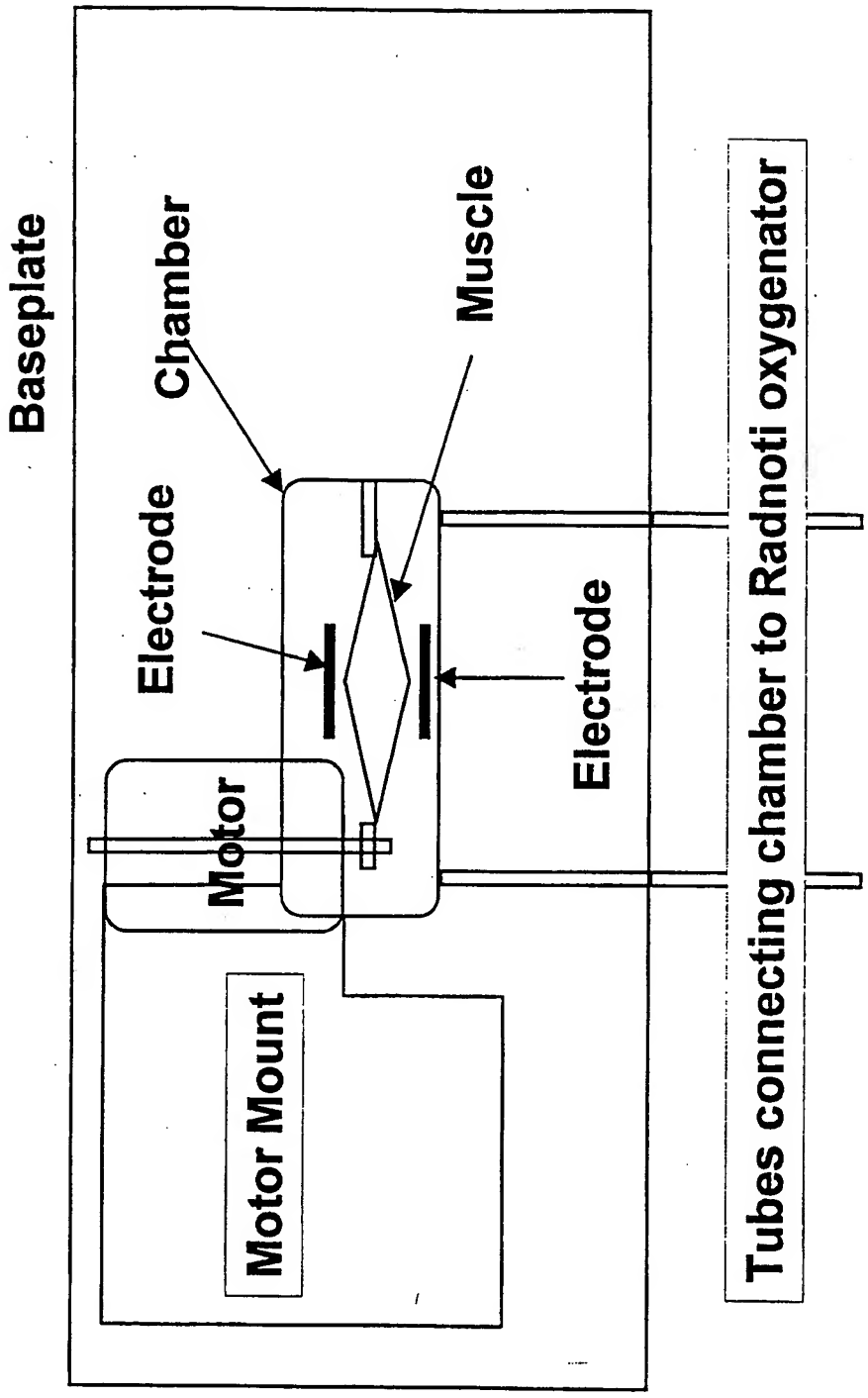

Baseplate
Chamber
Muscle
Electrode
Electrode
Motor
Motor Mount
Tubes connecting chamber to Radnoti oxygenator

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04367

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL : 514/44; 536/23.1, 23.5, 24.1; 435/320.1, 325, 455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1, 23.5, 24.1; 435/320.1, 325, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	HOLT, K.H. et al. Functional rescue of the sarcoglycan complex in ht BIO 14.6 hamster using delta-sarcoglycan gene transfer. Molecular Cell. May 1998, Vol. 1, pp. 841-848, see entire document.	1-20
Y	DUGGAN, D.I. et al. Autosomal recessive muscular dystrophy and mutations of the sarcoglycan complex. Neuromuscular disorders. 1996, Vol. 6, No. 6, pp. 475-482, see entire document.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 MAY 1999	Date of mailing of the international search report 09 JUN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RAM R SHUKLA Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04367

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	OGASAWARA, Y. et al. The use of heterologous promoters for adeno- associated virus (AAV) protein expression in AAV vector production. Microbiology Immunology. 1998, Vol. 42, No. 3, pp.177-185, see entire document.	3-11 and 15-20
Y,P	DUCLOS, F. et al. Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. The Journal of Cell Biology. 1998, Vol. 142, pp. 1461-1471, see entire document.	1-20
Y	VINCENT, C.K. et al. Different regulatory sequences control creatine kinase-M gene expression in directly injected skeletal and muscle. Molecular and Cellular Biology. February 1993, Vol. 13, No. 2, pp. 1264-1272, see entire document.	6-11, 14, 18, and 19
Y	BARLETT, R.J. et al. Long-term expression of a fluorescent reporter gene via direct injection of plasmid vector into mouse skeletal muscle: comparison of human creatine kinase and CMV promoter expression levels in vivo. Cell Transplantation. 1996, Vol. 5, No. 3, pp. 411-419, see entire document.	6-11. 14, 18, and 19
Y	MIZUNO, Y. et al. Sarcoglycan complex is selectively lost in dystrophic hamster muscle. American Journa of Pathology. February 1995, Vol. 146, No. 2, pp. 530-536, see entire document.	1-20
A,E	GREELISH, J.P. et al. Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. Nature Medicine. April 1999, Vol. 5, No. 4, pp. 439-443, see entire document.	1-20
Y	US 5,672,694 A (CAMPBELL et al.) 30 September 1997, see entire document.	1-20
Y,P	US 5,837,537 A (CAMPBELL et al) 17 November 1998, see entire document.	1-20
Y	US 5,260,209 A (CAMPBELL et al) 09 November 1993, see entire document.	1-20
Y	US 5,449,616 A (CAMPBELL et al) 12 September 1995, see entire document.	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04367

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 48/00; C12N 15/11; C07H 21/04; C12N 15/63; C12N 15/09, 15/85,86

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